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Quantitation of transient gene expression after electroporation

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ABSTRACT

The combination of a photometric reporter-gene assay, with transfection by electroporation, is potentially a rapid and sensitive tool for the study of genetic regulatory elements in many types of cells. We have found that the sensitivity, accuracy, and reproducibility of the technique is greatly improved by the inclusion of appropriately chosen carrier DNA as the primary DNA species present during electroporation. By using high levels of carrier, the activities of constructs of differing sizes can be quantitatively compared, active constructs can be assayed with sub-microgram amounts of plasmid, and the activities of the constructs are linear over a wide concentration of DNA. In addition, the activity of miniprep DNA can be screened without purification on CsCl gradients giving activities equal to CsCl-purified DNA. This is extremely useful when doing preliminary screening of large numbers of constructs for promoter or enhancer activities. We report the results of testing various types of DNA as carrier, and the parameters for optimizing its use.

INTRODUCTION

A number of recent studies suggest that electroporation is the method of choice for the introduction of DNA into cells of hemopoietic origin (1–4). These studies further document that transient transfection can be used to quantitate gene expression from transfected constructs, allowing the analysis of regulatory genetic elements. The development of expression vectors incorporating enzymes such as β-galactosidase (β-gal) (5–7) and luciferase (luc) (8) as reporter genes allows quantitation based on colorimetric or fluorescence assays. These increase the sensitivity and ease of quantitation, compared to the chloramphenicol transacetylase assay.

We have re-examined the parameters for gene expression by a number of human hematopoietic cell lines using a variety of viral and T-cell promoters and enhancers coupled to the luc reporter. Our principal aim was to find conditions that would allow the activity of related reporter constructs to be compared with confidence. We find that the use of appropriate carrier DNA during electroporation both satisfies this requirement and increases reproducibility of transient assay results. It also increases sensitivity of the luc reporter sufficiently, so that DNA from 1 ml minipreps may be screened without further purification. The use of a β-gal-expressing plasmid as an internal control to minimize sample-to-sample variation has also been evaluated.

MATERIALS AND METHODS

Cells

Peer (9) and Molt 13 (10) are human γ/δ T-cell lines, K562(S) is a human erythroleukemic cell line (11), BW5147 is a mouse α/β T-cell line (ATCC # TIB 47), and HD3 is a chicken avian erythroblastosis virus-transformed erythroid cell line (12). Peer, Molt 13, and BW5147 were maintained in RPMI 1640 supplemented with glutamine to a final concentration of 2 mM, 200 units of penicillin, and 0.1 mg of streptomycin/ml with 10% fetal bovine serum. K562 was maintained in Iscove's modified Dulbecco's medium supplemented as above. HD3 was grown in Iscove's modified Dulbecco's medium supplemented also as above, and containing 8% fetal bovine and 2% chicken serum. Cultures were split the day before use to bring them to a density of $0.5-1\times10^6$ at harvest. Molt 13 was used in all experiments except where noted.

Plasmids

Rous sarcoma virus (RSV)-luc, RSV-β-gal, and SV2-β-gal are the plasmids pRSVluc (8), pTB1 (7), and pSVβ-gal (6), respectively. Bluescript (KSII+) was obtained from Stratagene (La Jolla, CA). The following plasmids all contain restriction fragments derived from the T-cell receptor (TCR) α/δ locus (13), which are subcloned 5′ of the simian virus 40 minimal promoter in pSV232luc (8). pSV8.0 contains an 8-kb *Bam*HI/*Sal*I δ gene fragment which includes the δ gene enhancer (14; Showe *et al.*, unpublished results). Eα is a 6-kb *Bam*HI (α6.0) fragment, which includes the α gene enhancer (15; our unpublished results). 6.0PδM is the same fragment cloned 5′ of a TCR-δ variable gene promoter in pSV0luc. α1.5 is a 1.5-kb *Rsa*I/*Bam*HI fragment derived from a6.0 cloned in pSV232luc.

The following are the micrograms/pmol for each construct: RSV-luc = 4.3; RSV-β-gal = 5.2; SV2-β-gal = 5.2; SV232luc = 4.1; pSV8.0 = 9.3; Eα = 7.8; 6.0pδM = 13.6; pα1.5 = 6.5.

Electroporation

Transfection was carried out with a Gene Pulser (Bio-Rad, Richmond, CA) with a capacitance extender, using Gene Pulser disposable cuvettes with a 0.4 cm electrode gap containing a final volume of 0.3 ml of resuspended cells and added plasmid. This was the smallest volume found to give reproducible results in

this cuvette. Cells were centrifuged and resuspended in complete medium at approximately 1.8×10^7/ml. Expressed luc activity was found to be proportional to cell concentration when cells were resuspended over a range of at least $1-12\times10^7$/ml.

DNA (plasmids and carrier) was added to the cells in 0.1×TE (1 mM Tris chloride, pH 8.0, and 0.1 mM EDTA). The volume of this buffer was held constant at 50 µl for each transfection. The volume was adjusted so that the final transfection mixture contained 5×10^6 cells in 0.3 ml. Figures and tables in the text indicate micrograms of DNA added.

All operations were carried out at room temperature. As reported by Anderson *et al.* (4), we found no advantage in maintaining cells at a lower temperature either before or after transfection. DNA added after the voltage discharge made no contribution to transient expression. After transfection of a series of samples, the content of each cuvette was diluted to a final volume of 2 ml with fresh medium. Each sample was transferred to one of the 2.5-ml wells of a 24-well tissue culture plate (e.g. Falcon 3047), and the plate was incubated at 37°C. Cells were harvested for assay 18 to 24 hours after electroporation. Activity at 48 hours was found to be no higher.

Capacitance, voltage, and transfection medium were optimized using RSV-luc. For all cells we have tested, complete medium is optimal. For Molt 13, Peer, and K562, 960 mF at 200 volts (500 V/cm) gave optimal luc activity. The optimal voltage for HD3 and BW5147 was 250. Survival was 20 to 40%, based on trypan blue exclusion 18 to 24 hours after electroporation.

Assays for gene expression

Cells were harvested into 2.2 ml microcentrifuge tubes, centrifuged at 10,000 rpm for 2 min at 4°C, and the supernatant carefully removed by aspiration. The cell pellet is resuspended in 100 µl of cold lysis buffer (0.625% Triton X-100, 0.1 M potassium phosphate, and 1 mM dithiothreitol) at a pH of 7.8, and the resulting extract cleared by centrifugation at 10,000 rpm for 5 min. Half the reaction (50 µl) is assayed for luc activity (8) with a Monolight 2001 luminometer (Analytical Luminescence, San Diego, CA) set to integrate light output over a 10-second interval. Because the luminometer readings are not linear above 5×10^6 relative light units (RLU), any assay resulting in a value greater than 5×10^6 RLU is repeated using less extract, and the result extrapolated to 50 µl. When β-gal is used as an internal control, 10 to 50 µl of extract is assayed, as described by Rosenthal (16), except that the reactions are stopped by heating at 60°C for 10 min, since addition of the usual sodium carbonate stop buffer to the luc lysis buffer causes turbidity.

Activities are reported as RLU produced by 50 µl of cell extract, except when reported as specific activity (RLU/pmol), which is calculated from twice this value.

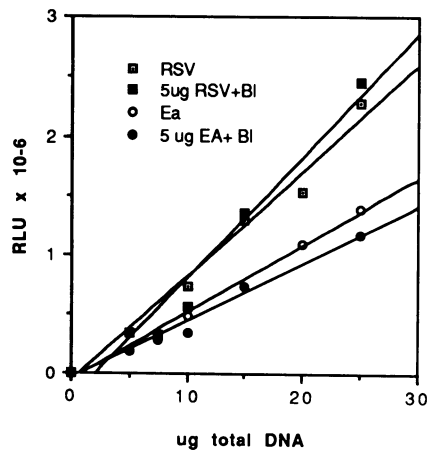

Figure 1. Luc reporter activity as a function of amount and type of DNA in electroporation cuvette. Increasing amounts of RSV-luc (RSV) or a TCR-α enhancer-luc (Eα) were transfected in a total volume of 0.3 ml (open symbols). For comparison, a constant 5 µg of reporter construct was transfected in the presence of increasing amounts of bluescript (Bl) as carrier (closed symbols).

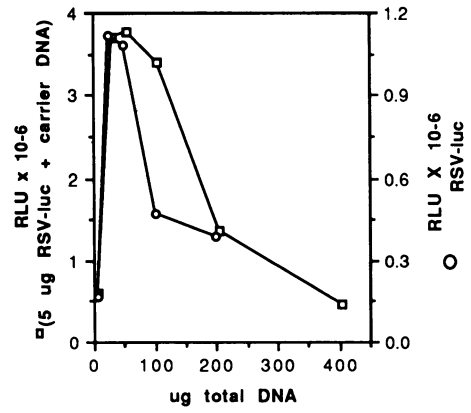

Figure 2. Maximum luc activity attained with reporter plasmid alone or with bluescript carrier DNA. RSV-luc was transfected alone at amounts between 5 and 200 µg (circles). Five µg RSV-luc was transfected with increasing amounts of bluescript carrier, up to 400 µg (squares).

Table 1. Comparison of bluescript with salmon sperm and *E. coli* DNA as carrier for transfection with 5 µg supercoiled RSV-luc.

Carrier DNA	Treatment	Size	Luc activity (light units x 10^{-6}) µg carrier added			
			0	5	10	20
None			0.167			
Bluescript	None	2.7 kb (circular)		0.34	0.60	1.29
Bluescript	*Bam*HI	2.7 kb (linear)		0.71	1.23	
Bluescript	*Bam*HI, denature	2.7 kb (single-stranded)		0.21	0.44	
Bluescript	Taq I	99 wt% > 0.4 kb		0.82	1.06	2.3
Bluescript	Sau3AI + Alu I	100 wt% < 0.3 kb 50 wt% > 0.2 kb		0.367	0.365	
Salmon sperm	Shear	>23 kb		0.186	0.175	0.126
Salmon sperm	Sau3AI	0.3 - 0.6 kb		0.31	0.32	0.36

All carrier was purified by two rounds of CsCl centrifugation. DNA treated with restriction enzymes was subsequently extracted with phenol-chloroform and ethanol precipitated.

Table 2. Comparison of bluescript with bacteriophage DNA as carrier for transfection with 5 µg supercoiled RSV-luc.

Carrier DNA	Treatment	Size	Luc activity (light units x 10^{-6}) µg carrier added			
			0	5	10	20
None			0.122 0.134			
Bluescript	None	2.7 kb		0.29	0.77	
Phage lambda	None	48 kb		0.038	0.0186	0.007
Phage lambda	*Xba*I	24 kb		0.091	0.064	
Phage lambda	*Pvu*I	9.5-12 kb		0.123	0.081	
Phage lambda	*Pst*I + *Eco*RI	5 bp-6 kb 94 wt% > 0.5 kb		0.180	0.428	
Phage T4	None	170 kb		0.0082		
Phage T4	Sonicate	250-600 bp		0.308	0.423	0.38

DNA was purified as described in Table 1. The sizes for untreated lambda and T4 DNA are those of the intact genome. The actual size was shown to be greater than 24 kb.

Preparation and quantitation of DNA

Plasmids for large-scale preparations were grown in *Escherichia coli* JM109 in terrific broth (17) by rapidly rotating an inoculum from a frozen stock or overnight culture in 500 ml of medium in a 2.1 flask for 60 hours (usually over a weekend). Plasmid DNA was isolated by alkaline lysis (18, p. 90). Miniprep plasmid DNA from 1 ml cultures was prepared by alkaline lysis (18, p. 368), with one additional phenol/chloroform extraction and two additional ethanol precipitations. All plasmids used as reporters or carrier were circular, and usually less than 10% nickel, unless otherwise noted. Bacteriophage lambda DNA was purchased from GIBCO BRL (Gaithersburg, MD). Bacteriophage T4 DNA was obtained by freezing and thawing phage particles in 0.5% sodium dodecyl sulfate in 1×1 mM Tris chloride, pH 8.0, and 0.1 mM EDTA, followed by phenol/chloroform extraction and ethanol precipitation. *E. coli* chromosomal DNA was obtained by lysozyme/EDTA lysis of *E. coli* JM109, followed by phenol/chloroform extraction and ethanol precipitation. All DNA preparations (except plasmid minipreps) were purified by two rounds of CsCl equilibrium centrifugation. When sonication was used to reduce the size of DNA, about 0.3 ml was treated at 0°C twice for 30 seconds with a Branson sonifier equipped with a microprobe. No attempt was made to determine the actual size of carrier DNA found by agarose gel electrophoresis to be greater than 24 kb. The concentration of CsCl-purified DNA was estimated spectrophotometrically at 280 nm, using the value of 1 AU=50 μg/ml. Miniprep DNA was estimated from band intensity on ethidium bromide-stained agarose gels.

Table 3. Effect of bluescript carrier on reproducibility of electroporation assayed by transient expression of the luc reporter.

		Transfect 50 μg construct			Transfect 0.5 pmol construct in 50 μg total DNA		
Construct	Number of preparations	Number of samples	Luc activity (RLU x 10^{-6}) Avg	SD	Number of samples	Luc activity (RLU x 10^{-6}) Avg	SD
SV232-luc	4	7	0.073	0.056	8	0.036	0.017
pSV8.0-luc	3	5	0.754	0.167	6	0.315	0.013
6.0 pδM-luc	3	6	1.732	0.461	6	0.563	0.017
RSV-luc	3	5	14.032	10.163	6	3.420	0.778

All of these assays were performed on the same day on the same batch of cells. Avg, average.

RESULTS

Construct DNA functions mainly as carrier during electroporation

When used in transient assays to measure levels of reporter gene expression, cells transfected by electroporation must be treated with 5 to 10 times the amount of DNA required by other techniques, such as lipofection or transfection using DEAE dextran (19). Chu *et al.* (20) reported that salmon sperm DNA could be used as carrier, but large amounts (up to 0.5 mg per assay) were required, and resulted in only a 2-fold increase in gene expression. To improve the sensitivity and reproducibility of transient expression assays of electroporated mammalian cells, we have examined a number of parameters which affect efficiency, and tested the effectiveness of DNA from various sources as carrier.

Fig. 1 shows luc reporter activity after transfection of Molt 13 cells by two different promoter-enhancer constructs. For each, increasing amounts of construct are compared to increasing amounts of bluescript carrier plasmid added to a constant 5 μg of construct. For both, increasing carrier has about the same effect as increasing construct. Fig. 2 shows that activity increases with added DNA, up to 50 to 100 μg (a concentration of 150 to 300 μg/ml) of either construct or carrier under our conditions, beyond which increased DNA interferes with gene expression.

Comparison of different types and sizes of carrier DNA

Using RSV-luc to transfect Molt 13, we have tested DNA from a number of sources as carrier to establish which combines effectiveness with low cost and ease of preparation. Table 1 shows

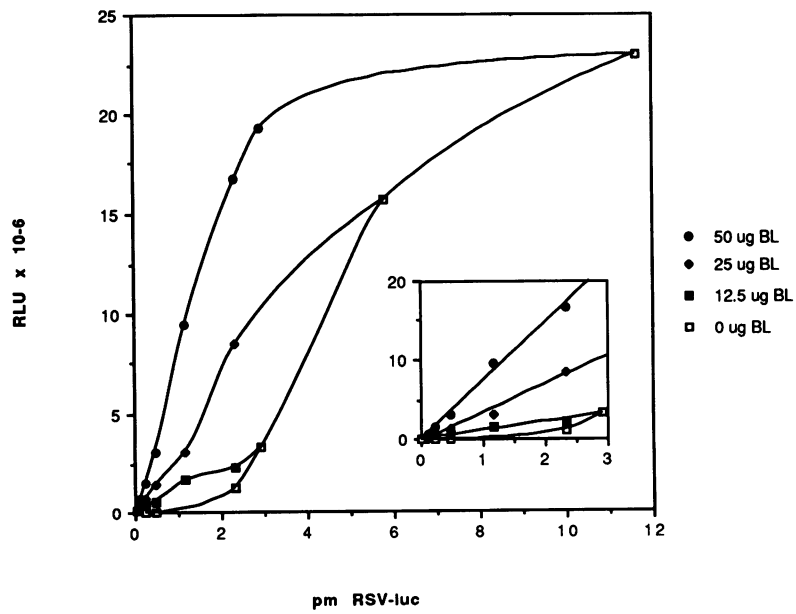

Figure 3. RSV-luc activity as a function of carrier bluescript level. Increasing amounts of RSV-luc were transfected either without added carrier (open squares), or with bluescript added to bring the total DNA in the electroporation cuvette to a constant 12.5 μg (closed squares), 25 μg (diamonds), or 50 μg (filled circles).

two experiments comparing the carrier efficiency of bluescript, salmon sperm, and *E. coli* DNA after a number of treatments with the activity of 5 μg of RSV-luc alone. The effectiveness of bluescript is not greatly changed if it is linearized or cleaved to fragments between 400 and 1400 bp with Taq I, but a further reduction of 200 to 300 bp diminishes its effectiveness, as does denaturation. Salmon sperm DNA is not effective as carrier, even if reduced to the size at which bluescript is most effective. A 2-fold increase in activity, as reported by Chu *et al.* (20), is the most we have found with this DNA. *E. coli* DNA sonicated to 0.6–2 kb was about twice as effective as sheared salmon sperm DNA (results not shown).

Comparisons of bluescript with salmon sperm and *E. coli* DNA as carrier suggest that DNA of low complexity, and a size from 0.4 to a few kb, is most effective. An extension of these studies with bacteriophage lambda and bacteriophage T4 DNA is consistent with this interpretation (Table 2). Both the 48-kb chromosome and 24-kb *Xba*I fragments of lambda phage inhibit expression of luc in the transient expression assay; the 9 to 12 kb *Pvu*I fragments are neutral, while the 0.5- to 6-kb *Pst*I/*Eco*RI fragments increase expression about half as well as bluescript. T4 DNA has not been extensively tested, but appears to be less effective than either bluescript or lambda DNA, even when reduced to the size at which these are most effective. Like other types of relatively complex DNA, it gives a 2- to 3-fold increase in activity at about 30 μg/ml, but increasing the carrier concentration further gives no further increase in transient expression.

The results described above suggest that up to 90% of the DNA used in electroporation can be replaced by carrier plasmid or phage DNA, which is relatively noncomplex and of a size of about 0.5 to 5 kb. The experiments described below were designed to optimize conditions for using bluescript or similar bacterial plasmid DNA as carrier.

A high ratio of carrier to construct DNA allows efficient quantitation of gene expression

Figs. 1 and 2 suggest that construct DNA has an effect as carrier on transient gene expression, independent of its inherent activity for mRNA production. This carrier effect of the construct depends not only on the purity of the plasmid but also on its size since, for constant moles of construct, activity is proportional to total DNA concentration. The results shown in Fig. 1 imply that, in the absence of added carrier, the molar activities of two plasmids with identical control elements (but different amounts of other DNA) will be proportional to their sizes. Meaningful comparison of the activities of two such plasmids requires that they be transfected at equal DNA concentrations, as well as equal numbers of moles.

To compare the molar activities of plasmids which differ in size, the variability in carrier effect attributable to the constructs can be minimized by making bluescript, or similar carrier, the principal DNA present during electroporation, and adjusting its amount to keep the total DNA concentration constant. Fig. 3 shows transfections carried out with increasing amounts of RSV-luc without carrier and at various levels of carrier. When carrier is used, the total DNA concentration for each RSV-luc series is held constant by the addition of bluescript. Without carrier (open squares), activity is not a linear function of construct transfected. The use of carrier both increases activity and provides a range over which activity is directly proportional to mol of construct. For RSV-luc and 50 μg total DNA, this range extends past 2 pmol of construct (about 20% of the total DNA), and the maximal activity at the end of the linear range is close to that obtained with 50 μg of construct alone. We have adopted 50 μg of bluescript carrier and 0.5 pmol of reporter construct as a standard assay condition, which provides good activity and is reasonably sparing of material.

Assay of transient transfections using a large excess of carrier DNA also increases the reproducibility obtained with different preparations of the same construct. Table 3 compares the activities and their variations for four constructs whose activities vary over a 200-fold range assayed both with and without carrier. The coefficient of variation (standard deviation/mean) was always less, in some cases 5 to 8 times less when carrier was used. Because the deviations for the samples determined with carrier are so much smaller, comparisons of constructs assayed with high levels of carrier are more reliable, and constructs of different sizes can be assayed under conditions of identical molar and total DNA concentrations.

Table 4. Electroporation of low levels of CsCl-purified and miniprep plasmid DNA compared.

Construct (purification)	pmol	(μg)	Luc activity RLU x 10^{-6}
RSV-luc (CsCl)	0.004	(0.017)	0.074
RSV-luc (CsCl)	0.020	(0.086)	0.334
RSV-luc (CsCl)	0.1	(0.430)	1.6
RSV-luc (CsCl)	0.5	(2.150)	6.0
pα1.5 (CsCl)	0.016	(0.1)	0.042
pα1.5 (CsCl)	0.032	(0.2)	0.072
pα1.5 (miniprep)	0.016	(0.1)	0.089
pα1.5 (miniprep)	0.032	(0.2)	0.147

All transfections contained 50 μg of bluescript DNA as carrier. Miniprep DNA concentration was estimated from an ethidium bromide-stained agarose gel by comparison to a standard DNA.

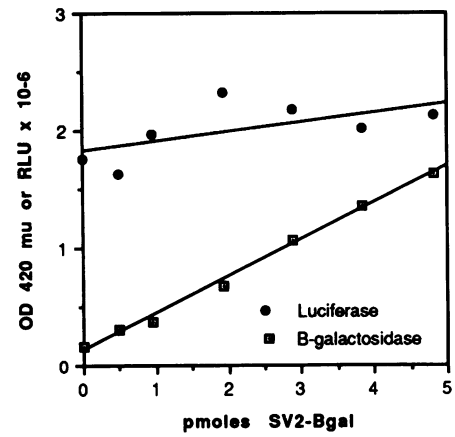

Figure 4. Activity of RSV-luc transfected in the presence of increasing SV2-β-gal. 10 mg of RSV-luc was transfected with increasing amounts of SV2-β-gal, the total amount of DNA held to a constant 50 μg by the addition of bluescript. Squares: β-gal activity; closed circles: luc activity.

Use of carrier allows screening of miniprep DNA

The use of carrier DNA allows the activity of relatively low amounts of construct to be accurately determined. If assay linearity holds at low construct amounts, it should be possible to screen miniprep DNA without further purification. Table 4

shows that the transfection assay using 50 μg of bluescript plasmid as carrier is linear over at least 3 orders of magnitude. Since the lowest value tested requires only nanograms of construct, we compared the activity of 0.1 to 0.2 μg of DNA prepared from a miniprep of a 1 ml culture with that purified as usual by centrifugation through two CsCl gradients. The specific activities of the two preparations were found to be comparable.

β-gal as an internal control

We have tested SV2-β-gal (6) and RSV-β-gal (7) with bluescript carrier as routine internal controls. Fig. 4 shows that as much as 5 pmol (25 μg) of SV2-β-gal can be transfected with 10 μg of RSV-luc in 50 μg total DNA without interfering with the luc expression. However, we routinely use only 0.1 to 0.5 pmol of β-gal reporter per assay for this purpose, depending on the level of expression of the construct in the cell line being used. RSV-β-gal has been tested extensively as an internal control for luciferase constructs in Molt 13 without evidence of interference (results not shown). It is also useful as a monitor of purity of miniprep DNA (data not shown).

Application to other cell lines and primary cells

In addition to Molt 13, we have tested RSV-luc, with bluescript as carrier, in a number of other cell lines with satisfactory results. For human Peer and K562, mouse BW5147, and chicken HD3 cells, the specific activities are 0.64, 23, 0.32, and 190×10^6 RLU/pmol, respectively. The assay for all of these cell lines was linear between 0.1 and 2.0 pmol of RSV-luc (data not shown).

Under the same conditions, normal human peripheral blood lymphocytes (stimulated with phytohemagglutinin and tested after 4 days) and 19-day chick embryo bone marrow cells give luc values comparable to, or higher than, Molt 13 (data not shown).

CONCLUSION

The finding that most of the reporter construct DNA used in electroporation transient transfection assays can be replaced by carrier facilitated the quantitative use of this technique. The requirement for less construct DNA minimizes variability due to differences in size or purity when constructs are compared. It may obviate the need for CsCl density gradient purification of test constructs altogether when using a sensitive reporter assay such as luciferase. The ability to assay miniprep DNA greatly simplifies screening many different constructs for activities. For promoter and cell combinations with specific activity approaching our RSV-luc/Molt 13 model (2×10^7 RLU/pmol), a few nanograms of construct DNA gives sufficient activity for accurate measurement. Even constructs 100- to 1000-fold less active may be accurately assayed using DNA from a 1-ml miniprep. Purity of carrier or any construct added in relatively high amounts is important for linearity of expression as a function of added reporter. A standard curve using RSV-luc or other reporter should be established with each new carrier DNA preparation.

It is likely that DNA from plasmids or small bacteriophages makes superior carrier because it lacks competing factor-binding sequences, which more complex DNA may contain. The relative insensitivity of carrier efficiency to carrier concentration of complex DNA could reflect a balance between protection of the construct on the one hand, and competition for factor binding sites on the other.

There is a strong size dependence for carrier efficiency, with an optimum between 0.5 and 5 kb for every DNA tested. This suggests that there might also be a size component to transfection efficiency of constructs themselves, which should be considered when testing constructs over 5 kb. Supercoiled circles might compensate for size by compactness, but transient expression from constructs as large as cosmids is likely to be low.

We have found co-transfection of a β-gal reporter internal control useful to correct for sample-to-sample variations on any given day, but inadequate to correct for day-to-day variation in target cells.

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